1981

Serological Groupings of Rhizobium Isolated from Native Legumes of South Dakota

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SEROLOGICAL GROUPINGS OF *RHIZOBIUM* ISOLATED FROM NATIVE LEGUMES OF SOUTH DAKOTA

BY

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A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science
Major in Microbiology

South Dakota State University
1981

ACKNOWLEDGMENTS

I wish to thank my husband, Ed, for his unfailing support, help, and love throughout the years associated with this program. Also a special thank you to my son, Eric, for his cheerful cooperation enabling his mother to study. My family's love is an inspiration to me always.

I would like to thank my major advisor, Dr. Robyn Hillam, for her direction, support, and patience. I would also like to thank Dr. Robert Pengra, department head, for his support and guidance. Both professors have been uniquely helpful to me and I appreciate their assistance.

The entire Microbiology faculty and staff have contributed to this effort, and I am grateful for their time and concern.

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INTRODUCTION

Nitrogen is an essential ingredient of all proteins, and, as such, is required by all forms of life. An inadequate supply of nitrogen for agriculture is therefore a contributing factor to global food shortages. In order to meet world food demands, research to increase useable nitrogen supplies for agriculture is becoming an urgent priority. This project, which combines the techniques of serology and nitrogen fixation research, provides an important contribution to this goal. This project expanded present knowledge of the rhizobia isolated from the native legumes of South Dakota.

Objective

This study was initiated to serologically identify and characterize the rhizobia from the following native legumes: *Astragalus flexuosus*, *Astragalus crassicarpus*, *Glycyrrhiza lepidota*, *Petalostemon purpureum*, and a wild legume recently introduced to the South Dakota prairies, *Astragalus cicer*. Since strains of *Rhizobium trifolii*, *Rhizobium meliloti*, *Rhizobium leguminosarum* and *Rhizobium phaseoli* from cultivated

legumes, as well as Agrobacterium tumefaciens, have been serologically characterized, they were included, for comparison purposes.

Background

Biological nitrogen fixation, the ability of a plant to fix atmospheric nitrogen into amino acids and proteins, offers a method to increase the supply of available nitrogen to plants and thereby decrease the dependence on nitrogenous fertilizers. Only a few genera of bacteria are able to fix nitrogen. These include blue-green algae and members of the genus Rhizobium. In order to fix nitrogen, rhizobia must exist in a symbiotic relationship with leguminous plants. Examples of this symbiosis are well-documented between species of the cultivated legumes and the genus Rhizobium. Since the rhizobium-legume symbiosis also restores nitrogen to the soil, legumes are frequently used in crop rotation and for reclamation of eroded and damaged land. Thus, the leguminous plants, in addition to being the most important commercial source of plant protein, serve other important roles in world food production. In an era of concern over declining energy resources, biological nitrogen fixation offers a means of increasing food production and soil

enrichment without encumbering the reserves of gas, coal, or oil for the costly production of nitrogenous fertilizers. Study of the genus Rhizobium, therefore, offers information potentially useful to agriculture and industry alike.

Serological relationships among the species of the genus Rhizobium have not been adequately studied, and therefore the number, the characteristics, and relative abundance of the different serogroups have not been determined. Only rhizobia from cultivated legumes have been extensively studied. Results from these studies on the commercial legumes have proven to be agriculturally and economically significant. These previous studies have, however, ignored the rhizobia from thousands of other legumes, including those native to South Dakota. Their potential value to agriculture has been largely ignored. Few, if any investigations, have been directed toward the serological relationships of the native legumes' rhizobia, and none on those from legumes native to South Dakota.

Rationale

Serological testing was chosen to study the rhizobia because of the increased specificity and sensitivity these methods offer compared with the more

traditional methods employed in soil microbiology. Antibody to each of the eight reference strains and eighteen native isolates was produced according to standard procedures. The antigen-antibody testing consisted of three steps: 1) cross-agglutination testing, to identify serological relationships among the isolates and to determine those suitable for further testing; 2) agglutination absorption procedures on selected strains and isolates, to define the extent of the serological relationships and to produce mono-specific antisera; and 3) indirect fluorescent antibody studies to identify the bacteroids from legume root nodules.

Significance

This study was directed toward obtaining results that would further expand the existing knowledge of the native legumes of South Dakota. The ability to serologically characterize rhizobia would be a potentially useful tool to quickly test native legumes on range/pasture land. This would enable the determination of both the possible presence and then subsequently identify the bacterial member of the successful rhizobium-legume symbiosis. Such a technique could eventually be applied to the reclamation of waste land. Further

applications of this study also include the acquisition of information about the serological relationships among the rhizobia from native legumes and those rhizobia from cultivated legumes. This information on the serological relationships of both groups could ultimately be used to improve the current classification scheme of the Rhizobiaceae.

LITERATURE REVIEW

1. The classification system of Rhizobiaceae

Biological nitrogen fixation is the process whereby atmospheric nitrogen is converted into useable plant protein. Only a few genera of bacteria, mainly the Rhizobium and the blue-green algae, are capable of nitrogen fixation. This process most frequently occurs as a result of the symbiotic relationship between the rhizobial cells and leguminous plants. The symbiotic relationship of the legumes and rhizobia has historically been used to classify these organisms.

1.1 The six recognized species

Most rhizobia isolated from different leguminous plants display markedly similar morphological and biochemical characteristics. Therefore, all rhizobia were considered to be a single species until the late 1800's (18). Rhizobia were later shown to differ in symbiotic preferences for plant groups. Baldwin and Fred (4) proposed five Rhizobium species based on this nodulation specificity. Subsequently, six species based on symbiotic preferences as well as litmus milk reaction were described in the third edition of Bergey's

Manual (7). The six species currently included in rhizobial classification are: Rhizobium trifolii Dangeard, Rhizobium phaseoli Dangeard, Rhizobium leguminosarum Frank, Rhizobium meliloti Dangeard, Rhizobium japonicum Kirchner, and Rhizobium lupini Schroeter. Each species of Rhizobium consisted of strains reciprocally nodulating all of the host plants within a certain cross-inoculation group, but not the host plants of other species of Rhizobium. Host plants for the six species of Rhizobium are the clover group (R. trifolii), the alfalfa and sweet clover group (R. meliloti), the bean group (R. phaseoli), the pea group (R. leguminosarum), the soybean group (R. japonicum), and the lupine group (R. lupini).

1.2 Problems with the present method of classification

This method of rhizobial taxonomy has been widely criticized (16, 41, 50, 51, 52) with the predominant criticism based on the symbiotic promiscuity of the rhizobia, that is, the ability of rhizobia from one species to nodulate plants from another cross-inoculation group. Plant inoculation tests investigating this symbiotic promiscuity have indicated additional examples of cross-inoculation groups of legumes. The

majority of these studies have concentrated on only the commercially important legumes. Results from these studies strongly dispute the present method of classification of Rhizobium.

An even greater limitation of this present method of classification is that, according to Norris (37) and Allen and Allen (3), only 8 - 10% of the known leguminous species have even been observed for possible nodulation. An even smaller percentage of species have had their rhizobia isolated and studied. It does not seem appropriate that a classification mechanism based on only a fraction of the organisms involved should be widely accepted.

This brief overview of the current classification scheme shows that there is considerable controversy and many problems associated with the present classification scheme of the genus Rhizobium.

1.3 Proposals for changes in the classification system

Proposals for changes in the classification system have been forthcoming. In these proposals, the speciation designations are based on standard methods of bacterial classification, including DNA base

composition (14, 15), DNA base homology (24), and numerical analysis (22, 34, 44).

A numerical analysis proposal by Graham (22) divided rhizobia into three groups: a fast-growing group, a slow-growing group, and R. meliloti. These groups were based on host plant preference, morphological, cultural, biochemical, and serological characteristics. Graham suggested that the first group, the fast-growing group, should include R. trifolii, R. leguminosarum, and R. phaseoli. The second group, the slow-growers, would consist of R. japonicum, R. lupini, and the cowpea rhizobia. However, since these slow-growers have DNA base composition, flagellation, and serological characteristics quite different from the fast-growers, Graham chose to place them into a separate genus and species, Phytomyxa japonicum. Graham's third group includes only R. meliloti and it has retained its present status as a species of Rhizobium. The major fault of this proposal is the continued omission of rhizobia from thousands of other legumes, including the native legumes. For instance, Abdel-Ghaffar and Jensen (1) conducted a study of the Lupinus densiflorus rhizobia that indicated that even some presently identified rhizobia may not fit into any of the presently proposed classification schemes. These

rhizobia exhibit characteristics common to both the fast-growing and slow-growing groups of rhizobia. This study further highlights the difficulty in classifying a genus where only a small percentage of species have been isolated and characterized.

2. Serological studies on Rhizobiaceae

2.1 Background

Serological studies on the rhizobia have been performed since 1913 (5), yet it was 1939-1940 before studies were used to arrange the family Rhizobiaceae into serological groups. Serological methods promise to be a valuable tool in studying the rhizobia due to the specificity and sensitivity of the antibody used in these techniques. Vincent and Waters considered serological methods "the best experimental tool for detailed studies between rhizobium strains in different environments" (48). Most research using serological methods has centered on the genus Rhizobium due to their commercial importance.

2.2 Classification studies on Rhizobium

The incorporation of serological methods in classification schemes offers many advantages.

Unfortunately, though the extensive serological cross-reactivity among the rhizobia will limit the ease with which these methods can be readily used. Serological data correlated with Graham's classification scheme grounds the six species of Rhizobium into three groups: the fast-growers, the slow-growers, and R. meliloti. Serogrouping determinations agreed with classifications based on biochemical characteristics (25), DNA base ratios (14, 49), plant inoculation patterns, and bacteriophage susceptibility (10, 32). Although each group has strains which exhibit unique serological properties, studies reveal extensive serological cross-reactivity among the groups (28, 43).

One set of studies report the cross-agglutination of R. trifolii, R. leguminosarum, and R. phaseoli by heterologous antisera. Twenty-one common antigens were recognized in a study involving R. trifolii and R. leguminosarum (28) and this provides the basis for this cross-agglutination. Other researchers have reported the agglutination of R. meliloti by antiserum prepared against species of the genus Agrobacterium, R. phaseoli, and R. leguminosarum. Additional cross-reactivity between various rhizobial groups and the genus Agrobacterium (21), and between rhizobium

lipopolysaccharide and pneumococcus antiserum (40) have been reported.

2.3 Classification studies on Agrobacterium

There appear to be few serological differences between A. tumefaciens and Rhizobium species. Cross-agglutination between Agrobacterium and R. meliloti has been well documented. The primary difference between these organisms is that A. tumefaciens is unable to nodulate legumes and fix nitrogen. It, therefore, is not surprising that many investigators are reporting a number of similarities between Agrobacterium and Rhizobium. These similarities include DNA base ratio, phage susceptibility, and serological similarities.

2.4 Minimal antigenic composition

The primary difficulty in serological characterization of the Rhizobiaceae is the lack of genus or group specific antigens. Vincent was the first to attempt the definition of the antigenic composition of R. trifolii (46), and subsequent researchers continue to find additional antigens (21, 34). R. meliloti is antigenically so heterogeneous that this species has been divided into thirty-nine serogroups (42). Because of the economic importance of soybeans, R. japonicum

has been extensively studied serologically (12, 43, 53), and yet the exact antigenic composition and number of serogroups in R. japonicum has not been determined.

2.5 Difficulties of serological characterization

It is apparent that there are several difficulties in using serological methods to study and classify the Rhizobium. The inability to define the minimal antigenic composition for any of the species or major groups is a serious problem. As cited earlier, other difficulties include the extreme cross-reactivity among the Rhizobium species and the small sample size used in most studies. Other obstacles frequently encountered in serological investigations of rhizobia were indicated by Vincent (47). Often after a strain has been isolated, and placed into a species category on the basis of plant nodulation/infective grouping tests, it will fail to react serologically with any of the standard test antisera available for that species. It is not unusual for bacteroids from nodules to fail to react with homologous antiserum prepared against the laboratory-grown plant inoculant. Several investigators have reported that nodules may contain both serologically reactive and non-reactive rhizobia. The inability for bacteroids to react may be due to an

antigenic shift between the bacteroid and the laboratory grown form. Because of these difficulties, Graham feels that it would be premature to propose a classification system based on serological properties until more studies have been completed and the methodology standardized (22).

2.6 Applications of serological methods

The application of serological methods to study rhizobia has contributed substantially to our knowledge of this organism. Several studies have produced insight into the ability of some strains to populate the soil and compete for root infectivity which ultimately results in nodule formation. Vincent and Hughes found that rhizobia isolated from different nodules on the same plant may be serologically different, but that only one serotype occurred within a nodule (26). Serological methods were used by Read (39) to identify the nodules successfully nodulated by the Rhizobium which had been used as a seed inoculum. Similar procedures were also used to determine the ability of different strains to compete for nodulation sites. A large number of bacteria were isolated from several plants (all plants of the same species located within a small area) and were characterized. It was found

that although one serotype may predominate, several other serotypes could also exist.

Efforts have been made to correlate the serotypes of successful strains of rhizobia with the area and soil type from which they were isolated. In a study that ascertained the dominant strains of R. japonicum that occurred in Iowa, it was found that one strain was able to predominate in several regions of the state (11). This occurrence was attributed to similar soil type and pH. It has also been demonstrated that the existence of a specific serotype is not limited to a given state, to a region of the country, or even to a continent (47). Comparative serological studies by many investigators have shown that there are marked antigenic similarities among the rhizobial strains belonging to the same species even though they were isolated from all over the world.

2.7 Potential uses of serological methods

Serological methodology for the characterization of rhizobia is still relatively undeveloped in terms of the potential applications. Such applications are varied, ranging from accumulating more data for classification to aiding agriculturists in their efforts to determine the best rhizobial inoculant for a

specified crop legume in a particular area. Such determinations could save the use of expensive fertilizers.

Similar information could also be utilized for the reclamation of waste land. Theoretically, the most successful reclamation will result from the use of native vegetation. Inclusion of native legumes in the plants chosen for reclamation would also restore nitrogen to the soil. However, background and plant inoculation information on the native legume rhizobia is lacking and serological data on native legumes and their rhizobia are almost non-existent.

Native prairie land and productive rangeland is a precious and diminishing resource in South Dakota. Serological research on the rhizobia and native legumes potentially vital to this land has not been done. Collaborative research by botanists and microbiologists could indicate the most successful native legume-rhizobium symbiotic pairs. The application of this information promises to enhance the potential for successful land reclamation.

This research project was a comparative serological study of reference Rhizobium strains and isolates from South Dakota native legumes. The intention of this project was to further define the

serological relationships among these strains and isolates. Since most of the native legume isolates were to be obtained from legumes located on native prairie, it was hoped that this study would also provide information which could ultimately be applied in programs designed to improve the quality of non-cultivated land within the state.

MATERIALS AND METHODS

1. Materials

1.1 *Rhizobium* and *Agrobacterium* strains and isolates used

Twenty-five *Rhizobium* cultures were selected for antisera production. Reference strains consisted of four cultures obtained from the American Type Culture Collection and four strains from the Nitragin Company. Seventeen isolates were cultured from root nodules of South Dakota native legumes and were representative of various geographical regions within the state. The native legume isolates were collected and tested using standard physiological and plant inoculation procedures by the graduate students and faculty of the Microbiology Department at South Dakota State University. The culture isolation procedure is detailed by Eide (19).

Antisera was also prepared against *A. tumefaciens*. This organism was isolated from a diseased tomato plant by the Plant Science Department at South Dakota State University.

1.2 Reasons for selecting strains and isolates

The eight reference strains represent four of the six species of Rhizobium described in the eighth edition of Bergey's Manual (7). The native isolates were selected to represent a variety of native legume plant groupings and include isolates from Glycyrrhiza lepidota, Astragalus flexuosus, A. crassicarpus, A. cicer, and Petalostemon purpureum plants. The list of strains, isolates, and sampling locations is found in Appendix I.

2. Antigen preparation

Whole cell or somatic entigen preparations were used for antisera production. Three to five-day-old actively motile cultures were used. The cultures were grown and maintained on the yeast mannitol agar (Medium 79) of Fred and Waksman as modified by Burton et al. (8). The composition of Medium 79 may be found in Appendix II. Antigen suspensions were prepared by growing cultures on Roux bottle slants and removing the growth from the surface by gentle agitation with sterile glass beads and physiological saline. The cell suspension was filtered twice through Whatman #3 filter paper to insure removal of agar. The turbidity of the filtered cell suspension was adjusted to obtain a

suspension of 5 x 10^6 cells/ml. For the strains used in this study, this corresponded to an O. D. of 0.39 at 550 nm on a Bausch and Lomb Spectronic 20 spectrophotometer.

3. Antiserum production

Increasing intravenous injection of the standardized antigen preparations were administered to healthy young rabbits according to the following schedule:

Day 0	Initial bleed and 0.5 ml antigen injected
Day 4	1.0 ml antigen injected
Day 8	2.0 ml antigen injected
Day 12	3.0 ml antigen injected
Day 21	2.0 ml antigen injected
Day 26	3.0 ml antigen injected
Day 35	Cardiac puncture bleed

All injections were administered intravenously in the marginal ear vein. Preimmunization bleeds were tested to assure that the rabbits did not have any preexisting titer. After the first cardiac puncture, repeat cardiac punctures were conducted at weekly intervals for a maximum of six bleeds. The serum titer of each

bleed was tested against the homologous antigen and booster injections of two to three ml were given if the titer fell substantially below the previous week's level.

4. Antigen-antibody testing

4.1 Cross-agglutination

Cross-agglutination tests were performed on all twenty-six isolates. The antiserum to each isolate was tested against antigen preparations of each of the twenty-five strains. A standard tube agglutination method was employed, using serial two-fold doubling dilutions of the serum, a constant volume of antigen, and carried out as far as the titer of the homologous system. The agglutination tubes were incubated six hours in a 37°C water bath, refrigerated overnight, then observed for agglutination. Saline plus antigen and saline plus serum controls were always included. These controls allowed the detection of any auto-agglutination, since cell preparations of the Rhizobiaceae show a marked tendency toward auto-agglutination.

4.2 Agglutinin adsorption

After the cross-agglutination testing was completed, adsorption procedures were carried out using selected isolates. Each antiserum selected for the adsorption procedure was separately adsorbed with each of the antigens showing a significant cross-reactivity (5% or greater of the homologous system titer) in the cross-agglutination testing.

The adsorption procedure outlined by Date and Decker was utilized, with the following modifications (12). Adsorbing antigen preparations contained 10×10^9 cells/ml and were prepared by concentrating the standard antigen preparation by centrifugation at 1000 r.p.m. for ten minutes. Equal volumes of serum and bacterial suspensions were mixed and incubated at 52°C in a water bath for four hours, and refrigerated overnight. The antigen-antibody complexes were removed by light centrifugation (1000 r.p.m. for three to five minutes), and the antiserum was thereafter stored in a refrigerator. This adsorption procedure was conducted on each homologous system to insure the removal of all agglutinins by this method and negative controls were included each time. The antiserum was titered after each adsorption by the tube agglutination method used in the cross-agglutination study. Frequently it was necessary

to repeat the adsorption procedure two or three times to eliminate the titer to a particular antigen. Monospecific antisera were then prepared for selected isolates by repeating the adsorption procedure with each cross-reacting system.

4.3 Indirect fluorescent antibody studies

One goal of this study was to attempt to determine serological relationships among rhizobia from native legumes by reacting the bacteroids of legume root nodules with various antisera. The microagglutination technique devised by Parker and Grove (38) proved to be unsatisfactory due to the insufficient amount of antigen contained in the tiny nodules of the native legumes. The indirect fluorescent antibody technique was selected as an alternative sensitive method to detect and identify rhizobia (antigen) in the root nodules.

4.3.1 Production of legume root nodules

Legume root nodules were obtained for the indirect fluorescent antibody tests by greenhouse cultivation of legumes. Plants selected for cultivation were the host plants of the rhizobia that were selected for continued study after the agglutinin adsorption studies

were completed. Seeds were obtained from the following sources:

1. Dollard cultivar Trifolium pratense and Travois cultivar Medicago sativa were obtained from the SDSU Foundation Seed Stock.
2. Glycyrrhiza lepidota, Astragalus flexuosus, Petalostemon purpureum, A. cicer and A. crassicarpus seeds were provided by the Plant Material Center, Soil Conservation Service, Bridger, Montana.
3. Commercial Northrup King Great Northern bean and Little Marvel pea seeds were used to grow host plants for Rhizobium phaseoli and R. leguminosarum inoculum.

The seeds were planted in a sterile mixture of 50% course sand and 50% soil contained in non-draining glazed crocks. Two crocks of each species were used as uninoculated controls. The seeds were surface-sterilized before planting by sequentially immersing them in 95% ethanol for one minute, undiluted commercial bleach for thirty minutes, and three one minute rinses of sterile distilled water. The seeds were allowed to dry at room temperature in sterile petri dishes with the lids slightly open (19). The seeds were placed

into the sand-soil mixture with the aid of sterile forceps to a depth of from one-quarter to one-half inch. After planting, each crock was watered with a nitrogen-free solution of plant nutrients. The plant nutrient solution was prepared by adding one gram of Bond's nitrogen-free modification of Crone's stock salt mixture (Appendix III) to one liter of deionized water (2). The components of this mixture were ground to a fine powder with a mortar and pestle. Thereafter until harvest, plants were watered periodically with deionized water. Plants were harvested when sufficient growth and greening indicated successful nodule formation. Depending on the species being studied, this varied from six to eight weeks. The plants were harvested by submerging the crock in water and carefully removing the intact root systems of the plants. Nodules were excised and indirect fluorescent antibody tests were conducted within twenty-four hours of harvest. The plants were kept moist after harvesting until testing could be completed. Most indirect fluorescent antibody testing was conducted immediately after harvesting.

4.3.2 Globulin labeling procedure

Indirect fluorescent antibody testing procedures consisted of reacting fluorescein isothiocyanate-labeled

porcine antirabbit IgG with antibody (absorbed antisera) and antigen (root nodule material). The porcine gamma globulin was labeled with the fluorescein isothiocyanate (FITC) according to the method of Cherry et al. (9). The protein content of the globulin preparation was determined by the standard method of Lowry et al. (35). The globulin solution was then adjusted to 1% by dilution with physiological saline. One-half molar carbonate-bicarbonate buffer, pH 9.0, was added to the chilled globulin in an amount equal to 10% by volume of the 1% globulin solution. (The composition of the buffers is given in Appendix IV.) The buffered globulin solution was chilled in an ice bath and FITC (0.05 mg FITC/mg protein in the solution) was added, with constant stirring, to the globulin solution. The product was stirred overnight in the cold. Unreacted FITC was removed by dialysis against frequent changes of physiological saline for two days. The product was then dialyzed against phosphate-buffered saline (0.01 M phosphate, pH 7.5) until a 100 ml beaker of the dialysate did not show fluorescence when viewed with ultraviolet light. The conjugate (FITC-labeled globulin) was cleared by centrifugation and merthiolate was added to a concentration of 1:1000. The conjugate was divided into 1 ml aliquots and frozen.

4.3.3 Indirect fluorescent antibody procedure

The indirect fluorescent antibody procedure was initially standardized using rhizobial stock cultures. Bacteroids from root nodules were then tested using the same procedure. When root nodules were used, they were crushed onto the fluorescent antibody (FA) slide with the aid of sterile forceps and large nodule debris was carefully removed. The smears were air dried, were fixed in 95% ethyl alcohol for one-two minutes, drained, and rinsed in FA buffer (Difco FA buffer), pH 7.2. The monospecific adsorbed antisera, diluted 1:100, was placed on the smear. Slides were then incubated for thirty minutes in a humid atmosphere and subsequently washed three times with FA buffer. The FITC-IgG conjugate (previously titered to determine optimal reactivity) was diluted to a concentration of 1:15 with physiological saline, was placed on smears, and incubated in a moist chamber at room temperature for thirty minutes. The slides were then sequentially rinsed again with FA buffer, then with three one minute rinses of distilled water, and then blotted with clean, absorbent paper. A small drop of mounting fluid (Difco FA mounting fluid) and coverslip were placed on each smear. The smear was then viewed with a fluorescent microscope.

RESULTS AND DISCUSSION

1. Cross-agglutination tests

1.1 Purpose and significance of results

Cross-agglutination tests were performed on each antiserum to the twenty-five strains and isolates. Results of these cross-agglutination tests were used for three purposes: 1) to obtain information on the serological relationships among the reference strains and native legume isolates; 2) to determine the strains and isolates most interesting for further study; and 3) to determine which strains and isolates should be used as adsorbing antigen to produce monospecific antisera.

In these tests, antiserum to each culture was tested against each of the other twenty-five antigen preparations. These tests demonstrated the existence of, or similarity in, cross-reactivity among the reference strains and isolates. It was assumed that positive cross-reactivity (the agglutination of one bacterium by antiserum produced against a different organism) would indicate the presence of an antigen common to the two cultures. Since the titer of the

homologous systems (the antigen and the corresponding antibody produced in response to that antigen) ranged from 1,280 to 81,920, it was necessary to relate the cross-reactive titers in such a way that the significance of the cross-reactive titer (as compared to the homologous titer) would be immediately apparent. For example, a cross-reactive or heterologous titer of 640 is more significant compared with a homologous titer of 1,280 than when compared with a homologous titer of 81,920. To simplify this comparison, the cross-reactive titers of each system were calculated as a percentage of the homologous titer, i.e., 5%, 10%, etc. Cross-reactivity of 5% or greater was considered significant. Table 1 presents the results on the cross-agglutination testing; the homologous system titers are reported numerically and the cross-reactive titers are reported as percentages.

1.2 Correlation of cross-agglutination and plant cross-inoculation tests

The results of the serological tests were then compared with the results of the plant cross-inoculation tests done by Eide (18). A positive correlation between these two testing procedures was deemed necessary in order for the serological data to be of value to the

Table 1

Antiserum \ Antigen	14480	127P17	127J11	127K19	14482	102F65	9930	128653	10004	WR1a(2)	WR3a(2)	V12(1)a	V36(1)	WR2b(2)	MPY	M1(2)YE	M2(3)YE	WR1(1)c	127J11c	FM 1(1)a	MFDb	ACP2	STS	PB5	MP7	PB1
R. trifolii ATCC 14480	10,240-20,480	20%																								
R. trifolii NC 127P17		10,240					5%						10%						10%							
Trifolium pratense L12(1)			10,240									10%	5%			10%	20%									
R. phaseoli NC 127K19				10,240																						
R. phaseoli ATCC 14482	10%	10%	5%		2560-5720			5%				5%									5%					
R. meliloti NC 102F65		10%	5%	10%		1280	10%					5%	5%			5%		5%								
R. meliloti ATCC 9930						5%	2,560-10,240																			10%
R. leguminosarum NC 128653	5%	5%						20,480	10%											5%						
R. leguminosarum ATCC 10004			5%		10%		5%	20%	1280-2560											10%	5%	5%				
Glycyrrhiza lepidota WR1a(2)		5%				20%	10%			1280	10% 640-1280		5%													
Glycyrrhiza lepidota WR3a(2)		20%	10%	20%		5%	10%	20%	10%			2560	20%			5%				5%		20%	20%			
Glycyrrhiza lepidota V12(1)a		10%	10%	5%								20%	5120			20%	20%			5%						
Glycyrrhiza lepidota V36(1)			10%				5%					20%	5120	1280-2560		20%	20%			20%	10%					
Glycyrrhiza lepidota M12b(2)		20%		10%		5%	5%	5%				5%	5%							20%	10%			20%		
Glycyrrhiza lepidota MPY		10%											5%		5120											
Astragalus flexuosus M1(2)YE			20%				10%					20%	10%			2560	20%									
Astragalus flexuosus M2(3)YE			20%									20%	10%			20%	5120			5%						
Astragalus flexuosus WR1(1)c				5%			5%							5%		5%		5120						5%		
Astragalus flexuosus 127J11c															10%					20,480	20%					
Astragalus flexuosus FM 1(1)a														20%						20%	10,240		5%			
Astragalus flexuosus MFDb																						10,240				
Astragalus crassicarpus ACP2			20%				5%	5%				10%			20%	5%				10%			1280-2560	5%		
Astragalus crassicarpus STS																							10%	10,240		
Astragalus cicer NC 985																									20,480	
Petalostemon purpureum MFP		10%					10%													5%						5120
Agrobacterium tumefaciens PB1								20%													20%					5120

agriculturist. The serological cross-reactivity was organized according to plant groupings, and compared with the plant cross-inoculation tests using the same plant groupings. The results of this comparison are found in Table 2. The correlation was rated according to the agreement of the cross-inoculation and serological testing. A positive correlation of 75%-100% was considered good; 50%-75% fair, and 0%-50% poor. A positive correlation was interpreted as evidence that the plant from which the organism was isolated was in fact its natural symbiont partner. Table 2 presents the correlation data.

2. Agglutinin adsorption studies

2.1 Criteria for selecting cultures for adsorption studies

Following the cross-agglutination studies, various strains and isolates were chosen for further study. The organisms selected for adsorption studies included: 1) organisms which demonstrated a good correlation of serological results with plant cross-inoculation results; 2) representatives of each plant grouping; and 3) as many typical reference strains as

TABLE 2 Comparison and Correlation of Plant Cross-Inoculation Tests with Serological Cross-Agglutination Tests

	Plant Group Infected									Degree of Correlation
	T.p.	P.v.	M.a.	P.s.	G.l.	A.f.	A.cr.	A.ci.	P.p.	
R. trifolii ATCC 14480	E/+	I/-	O/-	I/-	E/I/-	I/-	O/-	O/-	O/-	Fair
R. trifolii NC 127P17	E	N.t.	N.t.	N.t.	I	I	N.t.	N.t.	N.t.	Good
Trifolium pratense L12(1)	O	N.t.	N.t.	N.t.	E	I	N.t.	N.t.	N.t.	Good
R. phaseoli NC 127K19	O	E	O	I	O	O	O	O	O	Fair
R. phaseoli ATCC 14482	N.t./+	N.t./+	N.t./-	N.t./+	N.t./+	N.t./-	N.t./+	N.t./-	N.t./-	N.t.
R. meliloti NC 102F65	O/+	I/+	E/+	O/-	E/+	O/+	E/-	O/-	E/-	Fair
R. meliloti ATCC 9930	N.t./-	N.t./-	N.t./+	N.t./-	N.t./-	N.t./-	N.t./-	N.t./-	N.t./-	N.t./-
R. leguminosarum NC 128653	N.t./+	N.t./-	N.t./-	N.t./+	N.t./-	N.t./+	N.t./-	N.t./-	N.t./-	N.t.
R. leguminosarum ATCC 10004	O/-	I/+	O/+	E/+	E/-	I/+	E/-	O/-	O/-	Fair
Glycyrrhiza lepidota WR1a(2)	O/+	N.t./-	N.t./+	N.t./-	E/+	O/-	N.t./-	N.t./-	N.t./-	Fair
Glycyrrhiza lepidota WR3a(2)	O/+	N.t./+	N.t./+	N.t./+	E/-	O/+	N.t./+	N.t./-	N.t./-	Poor
Glycyrrhiza lepidota V12(1)a	O/+	N.t./+	N.t./-	N.t./-	E/+	O/+	N.t./-	N.t./-	N.t./-	Poor
Glycyrrhiza lepidota V34(1)	O/-	N.t./-	N.t./-	N.t./+	E/+	O/+	N.t./-	N.t./-	N.t./-	Fair
Glycyrrhiza lepidota N12b(2)	O/+	N.t./+	N.t./+	N.t./+	E/+	O/+	N.t./-	N.t./+	N.t./-	Poor
Glycyrrhiza lepidota MFY	E/E/+	O/-	O/-	O/-	I/E/+	I/E/-	O/-	I/-	E/-	Good
Astragalus flexuosus N1(2)YE	O/-	N.t./-	N.t./-	N.t./+	I/+	E/+	N.t./-	N.t./-	N.t./-	Good
Astragalus flexuosus N2(3)YE	O/-	N.t./-	N.t./-	N.t./-	I/+	E/+	N.t./-	N.t./-	N.t./-	Good
Astragalus flexuosus WR1(I)c	O/-	N.t./+	N.t./+	N.t./-	I/+	I/+	N.t./-	N.t./+	N.t./-	Good
Astragalus flexuosus 1171(I)c	O/-	N.t./-	N.t./-	N.t./-	O/+	E/+	N.t./-	N.t./-	N.t./-	Fair
Astragalus flexuosus FM 1(I)a	O/-	N.t./-	N.t./-	N.t./-	I/+	E/+	N.t./+	N.t./-	N.t./-	Good
Astragalus flexuosus MFDb	E/E/-	O/-	O/-	O/-	I/O/-	I/E/-	O/-	O/-	E/-	Fair
Astragalus crassicarpus ACP2	O/-	I/-	O/+	O/+	E/+	O/+	E/+	I/-	O/-	Poor
Astragalus crassicarpus 6TS	O/-	I/-	O/-	O/-	O/E/-	O/-	E/-	I/-	E/-	Fair
Astragalus cicer 9B5	O/-	O/-	O/-	O/-	I/-	O/-	O/-	E/-	E/-	Fair
Petalostemom purpureum MFP	O/+	I/-	O/+	O/-	O/-	E/+	E/-	I/-	E/-	Poor

N.t. = Not tested
E = Effective nodules
I = Ineffective nodules
O = No nodules present
T.p. = Trifolium pratense
P.v. = Phaseolus vulgaris
M.a. = Medicago sativa
G.l. = Glycyrrhiza lepidota
A.f. = Astragalus flexuosus
A.cr. = Astragalus crassicarpus
A.ci. = Astragalus cicer
P.p. = Petalostemom purpureum
\+ = Significant serological cross-reactivity with other strains and isolates from this plant
\- = Significant serological cross-reactivity not present with other strains and isolates from this plant

possible. Using these criteria, the cultures selected were:

1. Rhizobium trifolii ATCC 14480
2. Rhizobium trifolii NC 127P17
3. Rhizobium sp. L12(1)
4. Rhizobium phaseoli ATCC 14482
5. Rhizobium phaseoli NC 127K19
6. Rhizobium meliloti ATCC 9930
7. Rhizobium leguminosarum ATCC 10004
8. Rhizobium sp. WR1a(2)
9. Rhizobium sp. V34(1)
10. Rhizobium sp. N12b(2)
11. Rhizobium sp. N2(3)YE
12. Rhizobium sp. WR1(I)c
13. Rhizobium sp. FM1(I)a
14. Rhizobium sp. ACP2
15. Rhizobium sp. 9B5
16. Rhizobium sp. MFP
17. Agrobacterium tumefaciens PSI

All of these strains and isolates were chosen for subsequent adsorption studies because they fulfilled one or more of the criteria.

The organisms for which there was good correlation between cross-agglutination and plant

cross-inoculation tests included the following strains and isolates:

1. Rhizobium trifolii NC 127P17
2. Rhizobium sp. L12(1)
3. Rhizobium sp. N2(3)YE
4. Rhizobium sp. WR1(I)c
5. Rhizobium sp. FM1(I)a

Isolates added in order to adequately represent all of the plant groupings were:

1. Rhizobium sp. WR1a(2)
2. Rhizobium sp. V34(1)
3. Rhizobium sp. N12b(2)
4. Rhizobium sp. ACP2
5. Rhizobium sp. 9B5
6. Rhizobium sp. MFP

The following list of typical reference strains was also included. Any of the reference strains used in the cross-agglutination testing but not included in this group were eliminated because of their atypical cross-reactivity patterns. The exception to this is R. leguminosarum NC 128G53. This strain was not available. These considerations limited the reference strains to be used for further study to:

1. R. trifolii ATCC 14480
2. R. trifolii NC 127P17

3. *R. phaseoli* ATCC 14482

4. *R. phaseoli* NC 127K19

5. *R. meliloti* ATCC 9930

6. *R. leguminosarum* ATCC 10004

7. *A. tumefaciens* PSI

Seven isolates were included in these studies for reasons other than those previously defined. Initial studies with *Rhizobium sp.* L12(1) indicated it was an interesting isolate since it would not re-nodulate *Trifolium pratense*, its host plant, but would effectively nodulate the native legume, *Glycyrrhiza lepidota*. The three *A. flexuosus* isolates and three *G. lepidota* isolates were selected since they were isolated from the same plant but in different geographical regions of the state.

2.2 Purpose and results of adsorption studies

Results from the adsorption studies were used for three purposes: 1) to eliminate the strains and isolates not suitable for greenhouse testing; 2) to obtain monospecific antisera to be used for rapid serological differentiation of nodule-isolated bacteroids by the indirect fluorescent antibody procedure; and 3) to suggest serological relationships of the cultures both within and between the plant groups.

The results of the adsorption testing are found in Table 3. The homologous antiserum for each culture tested was adsorbed with each cross-reactive antigen (culture). The resulting adsorbed antiserum was then retitered with each cross-reactive antigen. From this information, it was possible to select the cultures necessary for complete adsorption of the serum in order to obtain a monospecific antiserum.

Satisfactory monspecific antisera for *R. trifolii* ATCC 14480 and *G. lepidota* V34(1) could not be produced. After adsorption with cross-reactive antigens, these sera had such a low homologous titer that they were unsuitable for further testing.

Using the previously described methods, fifteen different monospecific antisera were satisfactorily produced. The antisera and adsorbing antigens necessary for the production of the monospecific antisera are:

	Antiserum	Adsorbing Antigen(s)
1.	*R. trifolii* NC 127P17	*R. trifolii* ATCC 14480, *R. leguminosarum* NC 128G53, and *A. flexuosus* FM1(I)a
2.	*T. pratense* L12(1)	*A. flexuosus* N1(2)YE
3.	*R. phaseoli* ATCC 14482	*R. leguminosarum* NC 128G53
4.	*R. phaseoli* NC 127K19	None needed
5.	*R. leguminosarum* ATCC 10004	*T. pratense* L12(1)

TABLE 3 Residual Cross-Agglutination Titers of Selected Antisera After Specific Adsorptions

Rhizobium trifolii NC 127P17

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	127P17	V34	128G53	FM 1(1)a	14480
127P17(127P17)	< 80	< 80	< 80	< 80	< 80
127P17(V34)	1280	< 80	320	< 80	640
127P17(128G53)	10,480-20,480	< 80	< 80	320-640	1280
127P17(FM)	1280	< 80	320	< 80	640
127P17(14480)	640	320	320-640	320-640	< 80

TABLE 3 (cont'd.)

Trifolium pratense L12(1)

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	L12(1)	V34(1)	N2(3)YE	N1(2)YE	V12(1)a
L12(1)(L12(1))	< 80	< 80	< 80	< 80	< 80
L12(1)(V34(1))	2560	< 80	640	1280	640-1280
L12(1)(N2(3)YE)	320	160-320	80	< 80	< 80
L12(1)(N1(2)YE)	320	< 80	< 80	< 80	< 80
L12(1)(V12(1)a)	640-1280	160-320	160-320	320	< 80

TABLE 3 (cont'd.)

Rhizobium phaseoli ATCC 14482

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	14482	14480	ACP2	127P17	V12(1)a	L12(1)	128G53
14482(14482)	< 80	<80	<80	< 80	< 80	< 80	<80
14482(14480)	320	< 80	<80	320	<80	160	80-160
14482(ACP2)	1280	160	<80	160	<80	< 80	160
14482(127P17)	1280	320-640	<80	<80	<80	<80	80-160
14482(V12(1))	1280	<80	80-160	<80	<80	<80	160-320
14482(L12(1))	1280	< 80	<80	<80	<80	<80	320
14482(128G53)	1280	< 80	<80	< 80	<80	< 80	<80

TABLE 3 (cont'd.)

Rhizobium leguminosarum ATCC 10004

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	10004	L12(1)	14482	9930	128G53	1171(I)c	FM1(I)a	MFDb
10004(10004)	< 80	< 80	< 80	< 80	<80	< 80	< 80	< 80
10004(L12(1))	1280-2560	< 80	< 80	< 80	<80	< 80	< 80	< 80
10004(14482)	640	< 80	< 80	< 80	<80	160	320	< 80
10004(9930)	1280	<80	80-160	<80	<80	160-320	160	< 80
10004(128G53)	640	<80	< 80	160	<80	< 80	< 80	< 80
10004(FM 1(I)a)	1280-2560	<80	80-160	<80	< 80	< 80	< 80	< 80
10004(MFDb)	2560	<80	< 80	<80	320-640	< 80	< 80	< 80
10004(1171(I)c)	1280	<80	80-160	<80	< 80	< 80	< 80	< 80

TABLE 3 (cont'd.)

Rhizobium meliloti ATCC 9930

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	9930	102F65	PSI
9930(9930)	< 80	< 80	< 80
9930(102F65)	2560	< 80	< 80
9930(PSI)	2560	< 80	< 80

Glycyrrhiza lepidota WR1a(2)

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	WR1a(2)	127P17	102F65	9930	WR3a(2)	V34(1)
WR1a(2)(WR1a(2))	< 80	< 80	< 80	< 80	< 80	< 80
WR1a(2)(127P17)	640	< 80	160	320	160	< 80
WR1a(2)(102F65)	160	< 80	< 80	< 80	160	< 80
WR1a(2)(9930)	160	< 80	< 80	< 80	160	< 80
WR1a(2)(WR3a(2))	320	< 80	80	160	< 80	< 80
WR1a(2)(V34(1))	160	< 80	160	160	160	< 80

TABLE 3 (cont'd.)

Glycyrrhiza lepidota N12b(2)

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	N12b(2)	127P17	127K19	102F65	9930	128G53	V12(1)	V34(1)	1171(I)c	FM1(I)a	9B5
N12b(2)(N12b(2))	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80
N12b(2)(127P17)	640	< 80	< 80	< 80	< 80	< 80	< 80	< 80	320	320	< 80
N12b(2)(127K19)	320-640	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80
N12b(2)(102F65)	1280	< 80	< 80	< 80	< 80	< 80	< 80	< 80	320	320	< 80
N12b(2)(9930)	1280	< 80	< 80	< 80	< 80	< 80	< 80	< 80	160	160	160
N12b(2)(128G53)	1280	< 80	< 80	< 80	< 80	< 80	< 80	< 80	320	320	< 80
N12b(2)(V12(1)a)	1280	160	< 80	< 80	< 80	< 80	< 80	< 80	160	160	< 80
N12b(2)(V34(1))	640	< 80	< 80	< 80	< 80	< 80	< 80	< 80	320	640	< 80
N12b(2)(1171(I)c)	1280	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80
N12b(2)(FM1(I)a)	640	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80	< 80
N12b(2)(9B5)	1280	< 80	< 80	< 80	< 80	< 80	< 80	< 80	320	160	< 80

TABLE 3 (cont'd.)

Astragalus flexuosus N(3)YE

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	N2(3)YE	L12(1)	V12(1)a	V34(1)	N1(2)YE	1171(I)c
N2(3)YE(N2(3)YE)	< 80	< 80	< 80	< 80	< 80	< 80
N2(3)YE(L12(1))	640-1280	< 80	< 80	< 80	320-640	< 80
N2(3)YE(V12(1)a)	640	< 80	< 80	< 80	640	320
N2(3)YE(V34(1))	2560	640	80-160	< 80	640-1280	< 80
N2(3)YE(N(2)YE)	160	< 80	< 80	< 80	< 80	< 80
N2(3)YE(1171(I)c)	2560	640	160	160	1280	< 80

Astragalus flexuosus WR1(I)c

Antiserum Adsorbed (Adsorbing Antigen) \ Antigen	WR1(I)c	127K19	9930	N12b(2)	N1(2)YE	9B5
WR1(I)c(WR1(I)c)	< 80	< 80	< 80	< 80	< 80	< 80
WR1(I)c(127K19)	160	< 80	160	< 80	< 80	< 80
WR1(I)c(9930)	80-160	< 80	< 80	< 80	< 80	< 80
WR1(I)c(N12b(2))	320	< 80	320	< 80	< 80	< 80
WR1(I)c(N1(2))	160	< 80	160	< 80	< 80	< 80
WR1(I)c(9B5)	320	< 80	320	< 80	< 80	< 80

TABLE 3 (cont'd.)

Astragalus flexuosus FM1(I)a

Adsorbed Antiserum (Adsorbing Antigen) \ Antigen	FM1(I)a	N12b(2)	1171(I)c	ACP2
FM (FM)	< 80	< 80	< 80	< 80
FM (N12b)	5120	< 80	2560	< 80
FM (117#)	160-320	80	< 80	< 80
FM (ACP2)	2560	320-640	1280-2560	< 80

TABLE 3 (cont'd.)

Astragalus crassicarpus ACP2

Adsorbed Antiserum (Adsorbing Antigen) \ Antigen	ACP2	L12(1)	9930	128G53	V12(1)a	V34(1)	N1(2)YE	1171(I)c	6TS	MFP
ACP2(ACP2)	<80	< 80	< 80	< 80	< 80	<80	< 80	< 80	< 80	< 80
ACP2(L12(1))	320	< 80	< 80	< 80	< 80	80-160	< 80	< 80	< 80	< 80
ACP2(9930)	320-640	< 80	< 80	< 80	< 80	< 80	< 80	80-160	< 80	< 80
ACP2(128G53)	80-160	80-160	80-160	< 80	< 80	80-160	< 80	160	< 80	80-160
ACP2(V12(1)a)	320-640	160-320	< 80	< 80	< 80	160-320	< 80	< 80	< 80	< 80
ACP2(V34(1))	320	< 80	< 80	< 80	< 80	<80	< 80	< 80	< 80	< 80
ACP2(N1(2)YE)	1280	320	< 80	< 80	< 80	<80	< 80	< 80	< 80	< 80
ACP2(1171(I)c)	160-320	160	< 80	< 80	< 80	320-640	< 80	< 80	< 80	< 80
ACP2(6TS)	160-320	80-160	80-160	< 80	< 80	160	< 80	< 80	< 80	< 80
ACP2(MFP)	320	80-160	< 80	< 80	< 80	160-320	< 80	80-160	< 80	< 80

TABLE 3 (cont'd.)

Petalostemon purpureum MFP

Adsorbed Antiserum (Adsorbing Antigen) \ Antigen	MFP	127P17	9930	1171(I)c	ACP2	9B5
MFP(MFP)	< 80	< 80	< 80	< 80	< 80	< 80
MFP(127P17)	320-640	< 80	320	< 80	< 80	< 80
MFP(9930)	160	< 80	< 80	< 80	< 80	< 80
MFP(1171(I)c)	640-1280	< 80	< 80	< 80	< 80	80-160
MFP(ACP2)	80-160	< 80	320	160	< 80	< 80
MFP(9B5)	640	< 80	80-160	< 80	< 80	< 80

Agrobacterium tumefaciens PSI

Adsorbed Antiserum (Adsorbing Antigen) \ Antigen	PSI	128G53	MFDb
PSI(PSI)	< 80	< 80	< 80
PSI(128G53)	1280	< 80	< 80
PSI(MFDb)	1280	< 80	< 80

6.	*R. meliloti* ATCC 9930	*R. meliloti* NC 102F65
7.	*G. lepidota* WR1a(2)	*R. meliloti* NC 192F65 and *G. lepidota* WR3a(2)
8.	*G. lepidota* N12b(2)	*A. flexuosus* 1171(I)c
9.	*A. flexuosus* N2(3)YE	*T. pratense* L12(1)
10.	*A. flexuosus* WR1(I)c	*R. meliloti* ATCC 9930
11.	*A. flexuosus* FM1(I)a	*A. flexuosus* 1171(I)c
12.	*A. crassicarpus* ACP2	*G. lepidota* V34(1)
13.	*A. cicer* NC 9B5	None needed
14.	*P. purpureum* MFP	*R. trifolii* NC 127P17 and *R. meliloti* ATCC 9930
15.	*A. tumefaciens* PSI	*R. leguminosarum* NC 128G53 and *A. flexuosus* MFDb

2.3 Reciprocal adsorption relationships

The initial cross-agglutination testing demonstrated the existence of serological relationships among the cultures. The true extent of these relationships, however, could only be determined after adsorption tests. For instance, if a particular culture being used to adsorb a heterologous antiserum either eliminated or substantially reduced the cross-reactivity of another cross-reactive isolate, it was assumed that the two shared a common antigenic component, and therefore indicated some serological similarity. Such an occurrence is called reciprocal adsorption. The greater

the number of shared antigenic components, the stronger this serological relationship; therefore, when reciprocal adsorption was observed two or more times, it was considered significant. Table 4, organized according to plant grouping, lists the instances of reciprocal adsorption which were encountered. Several serological relationships among the strains and isolates using the adsorption data and Table 4 can be determined.

Serological relationships of the organisms nodulating commercially important legumes and native isolates were derived from the cross-agglutination and adsorption tests, and reciprocal adsorption patterns. These results are discussed below.

2.3.1 *Trifolium pratense*

2.3.1.1 *R. trifolii* NC 127P17

The homologous titer of *R. trifolii* NC 127P17 was reduced most by another *R. trifolii* isolate, ATCC 14480. Since these two organisms were isolated from the same host plant, this observation was expected. The relationships of *R. trifolii* with *R. phaseoli* and *R. leguminosarum* has been previously documented (42). The reciprocal adsorption information gained through this investigation substantiated these

TABLE 4

Strains and Isolates Demonstrating
Reciprocal Adsorption
(Grouped According to Host Plant Nodulation)

1. *Trifolium pratense*

   R. trifolii NC 127P17 and *R. meliloti* NC 102F65

   R. trifolii NC 127P17 and *R. meliloti* ATCC 9930*

   R. trifolii NC 127P17 and *R. leguminosarum* NC 128G53

   R. trifolii NC 127P17 and *G. lepidota* V12(1)a

   R. trifolii NC 127P17 and *A. flexuosus* 1171(I)c

   R. trifolii NC 127P17 and *A. cicer* NC 9B5

   Rhizobium sp. L12(1) and *R. phaseoli* ATCC 14482

   Rhizobium sp. L12(1) and *R. meliloti* ATCC 9930

   Rhizobium sp. L12(1) and *R. leguminosarum* NC 128G53

   Rhizobium sp. L12(1) and *A. flexuosus* 1171(I)c

2. *Phaseolus vulgaris*

   R. phaseoli NC 127K19 and *R. meliloti* ATCC 9930

   R. phaseoli NC 127K19 and *A. cicer* NC 9B5

   R. phaseoli ATCC 14482 and *T. pratense* L12(1)

3. *Medicago sativa*

   R. meliloti ATCC 9930 and *R. meliloti* NC 102F65*

   R. meliloti ATCC 9930 and *R. trifolii* NC 127P17*

   R. meliloti ATCC 9930 and *T. pratense* L12(1)

   R. meliloti ATCC 9930 and *R. phaseoli* NC 127K19

   R. meliloti ATCC 9930 and *R. leguminosarum* NC 128G53

   R. meliloti ATCC 9930 and *G. lepidota* V12(1)a

TABLE 4 (cont'd.)

R. meliloti ATCC 9930 and *G. lepidota* V34(1)

R. meliloti ATCC 9930 and *A. flexuosus* N1(2)YE

R. meliloti ATCC 9930 and *A. flexuosus* 1171(I)c

R. meliloti ATCC 9930 and *A. flexuosus* FM 1(I)a

R. meliloti NC 102F65 and *G. lepidota* V34(1)

4. *Pisum sativum*

R. leguminosarum NC 128G53 and *R. trifolii* NC 127P17

R. leguminosarum NC 128G53 and *T. pratense* L12(1)

R. leguminosarum NC 128G53 and *R. meliloti* ATCC 9930

R. leguminosarum NC 128G53 and *G. lepidota* V12(1)a

R. leguminosarum NC 128G53 and *G. lepidota* V34(1)

R. leguminosarum NC 128G53 and *A. flexuosus* 1171(I)c

R. leguminosarum NC 128G53 and *A. flexuosus* FM 1(I)a

R. leguminosarum NC 128G53 and *A. flexuosus* MFDb

5. *Glycyrrhiza lepidota*

G. lepidota V12(1)a and *R. trifolii* NC 127P17

G. lepidota V12(1)a and *R. meliloti* ATCC 9930

G. lepidota V12(1)a and *R. leguminosarum* 128G53*

G. lepidota V12(1)a and *G. lepidota* V34(1)#13

G. lepidota V12(1)a and *A. flexuosus* N1(2)YE*

G. lepidota V12(1)a and *A. flexuosus* 1171(I)c

G. lepidota V34(1) and *R. meliloti* NC 102F65*

G. lepidota V34(1) and *R. meliloti* ATCC 9930

TABLE 4 (cont'd.)

G. lepidota V34(1) and R. leguminosarum NC 128G53*

G. lepidota V34(1) and A. flexuosus N1(2)YE*

G. lepidota V34(1) and A. flexuosus 1171(I)c*

G. lepidota V34(1) and A. flexuosus FM 1(I)a

G. lepidota V34(1) and A. cicer NC 9B5

6. Astragalus flexuosus

A. flexuosus N1(2)YE and R. meliloti ATCC 9930

A. flexuosus N1(2)YE and G. lepidota V12(1)a*

A. flexuosus N1(2)YE and G. lepidota V34(1)*

A. flexuosus N1(2)YE and A. flexuosus N2(3)YE

A. flexuosus N1(2)YE and A. flexuosus 1171(I)c

A. flexuosus 1171(I)c and R. trifolii NC 127P17

A. flexuosus 1171(I)c and T. pratense L12(1)

A. flexuosus 1171(I)c and R. meliloti ATCC 9930

A. flexuosus 1171(I)c and R. leguminosarum NC 128G53*

A. flexuosus 1171(I)c and G. lepidota V12(1)a

A. flexuosus 1171(I)c and G. lepidota V34(1)*

A. flexuosus 1171(I)c and A. cicer 9B5

A. flexuosus FM 1(I)a and R. meliloti ATCC 9930

A. flexuosus FM 1(I)a and R. leguminosarum NC 128G53

A. flexuosus FM 1(I)a and G. lepidota V34(1)

A. flexuosus MFDb and R. leguminosarum NC 128G53

TABLE 4 (cont'd.)

7. Astragalus crassicarpus

   None

8. Astragalus cicer

   A. cicer NC 9B5 and R. trifolii NC 127P17

   A. cicer NC 9B5 and R. phaseoli NC 127K19

   A. cicer NC 9B5 and R. meliloti ATCC 9930

   A. cicer NC 9B5 and G. lepidota N12b(2)

   A. cicer NC 9B5 and A. flexuosus 1171(I)c

9. Petalostemon purpureum

   None

10. Agrobacterium tumefaciens

    None

* indicates 3 or more reciprocal adsorptions

relationships. This data further indicates that R. trifolii NC 127P17 appears to be related to two native isolates: A. flexuosus and G. lepidota, and to A. cicer.

2.3.1.2 T. pratense L12(1)

T. pratense isolate, L12(1) is the organism that would not re-nodulate T. pratense after initial isolation, but did effectively nodulate G. lepidota, and ineffectively nodulated A. flexuosus during the subsequent plant inoculation studies. Serological tests demonstrated strong reciprocal adsorption patterns with the A. flexuosus isolates but yet failed to demonstrate any reciprocal adsorption with isolates from either R. trifolii or G. lepidota. With this conflicting information, it is difficult to assign this isolate to any specific plant host. One possibility is that the natural plant host is an Astragalus species not used in this study.

2.3.2 Phaseolus vulgaris

The two reference R. phaseoli strains did not appear to be related either by data obtained through cross-agglutination tests or adsorption tests. Ironically, Table 4 indicates that R. phaseoli NC 127K19

is more closely related to R. meliloti ATCC 9930 and A. cicer NC 9B5, than to the other R. phaseoli strain. This relationship, however, is not supported by other studies on this species (28). The results also indicate that R. phaseoli ATCC 14482 is closely related to T. pratense isolate L12(1). On the basis of this information, it is difficult to include R. phaseoli into any particular serological group containing these other reference strains.

2.3.3 Medicago sativa

2.3.3.1 R. meliloti ATCC 9930

R. meliloti ATCC 9930 appears to share several antigens with several reference strains and native isolates. It appears to be most similar to the other R. meliloti strain, NC 102F65. The broad range of serological cross-reactivity with cultures isolated from other plant species does not, however, correlate with the data from the plant inoculation studies of most R. meliloti strains. The reduction in titer in the agglutination adsorption tests would therefore seem to indicate some common antigenic component found in many strains of rhizobia. It would appear that this antigenic component is present in many species and

strains of rhizobia, since so many isolates are reciprocally adsorbed with R. meliloti ATCC 9930.

2.3.4 Pisum sativum

2.3.4.1 R. leguminosarum NC 128G53 and R. leguminosarum ATCC 10004

R. leguminosarum NC 128G53 was also serologically similar to a broad range of both reference strains and native isolates. Its reactivity with R. trifolii and R. phaseoli reference strains has been previously documented, and would seem to verify it as a member of Graham's fast-growing group (22). Antiserum against R. leguminosarum NC 128G53 was not tested by adsorption, but this antigen was used in several adsorption schemes and frequently caused a reduction in titer. This strain, like R. meliloti ATCC 9930, may have an antigenic component common to many species. The homologous titer of R. leguminosarum ATCC 10004 was significantly reduced after adsorption with R. leguminosarum NC 128G53 and R. phaseoli ATCC 14482. This cross-reactivity would then indicate that R. leguminosarum ATCC 10004 is also a member of the fast-growing group.

The serological relationships of the cultures

from native legumes were of special interest in this study. These cultures will be discussed in detail.

2.3.5 Glycyrrhiza lepidota

2.3.5.1 G. lepidota V12(1)a and V34(1)

G. lepidota isolates V12(1)a and V34(1) were the only G. lepidota cultures to show reciprocal adsorption with other strains and isolates. V12(1)a was antigenically related to three reference strains, one G. lepidota culture, and two A. flexuosus cultures. V34(1) had a strong serological relationship with V12(1)a, several reference strains, one G. lepidota, and two A. flexuosus isolates. Isolates V34(1) and V12(1)a demonstrated the only reciprocal adsorption relationship noted among the G. lepidota isolates, and both also appear related to the same two A. flexuosus isolates. This serological data supports the suggestion by Eide that G. lepidota and A. flexuosus isolates belong in the fast-growing group suggested by several researchers (19). It is interesting to note that these two organisms, which bear a strong serological similarity to each other, were isolated from the same region. Since they have different cross-agglutination patterns, however, they are not the same organism.

2.3.5.2 G. lepidota WR1a(2) and WR3a(2)

G. lepidota WR1a(2) and WR3a(2) did not display complete reciprocal adsorption by each other but did show strong antigenic similarities in the adsorption system. The homologous titer of WR1a(2) was reduced after adsorption with WR3a(2), however, the titer was reduced to a greater extent by other strains and isolates. WR1a(2) and WR3a(2) did demonstrate some serological similarities in the initial cross-agglutination studies, but this relationship does not seem to be as strong as the V12(1)a and V34(1) relationship. After adsorption tests, the WR1a(2) isolate appeared to be most clearly related to the reference strain R. meliloti ATCC 9930. Graham has proposed that R. meliloti be considered a separate species, and yet Graham admitted that there was some cross-over between R. meliloti and the fast-growing group (22). Such considerations make it difficult to place WR1a(2) into a definite group. Plant cross-inoculation tests by Eide indicate that it is a member of the fast-growing group of rhizobia.

2.3.5.3 G. lepidota N12b(2)

G. lepidota N12b(2) was reciprocally adsorbed by the A. cicer strain NC 9B5. It did not appear closely related to the isolates of any other plant

group. This isolate appears to be somewhat different serologically than the other G. lepidota isolates. With only this data, it is difficult to include it in any of the serological groups at this time.

2.3.6 Astragalus flexuosus

2.3.6.1 A. flexuosus N1(2)YE and N2(3)YE

A. flexuosus isolates N1(2)YE and N2(3)YE appear closely related to each other. These two isolates had nearly identical cross-agglutination patterns, and this similarity was further substantiated by the adsorption data. N1(2)YE was more prevalent in reciprocal adsorption relationships than N2(3)YE. These two isolates appeared most similar to the other native isolates, such as those from A. flexuosus and G. lepidota, than to the reference strains. Due to the lack of information on native rhizobia, it is difficult to relate this to other classification schemes; however, this study indicates that isolates of A. flexuosus and G. lepidota should be in the same group.

2.3.6.2 A. flexuosus WR1(I)c

A. flexuosus isolate WR1(I)c did not show any reciprocal adsorption with other cultures, but the

homologous titer was reduced after adsorption with all cross-reactive antigens. It should also be placed into the fast-growing group with other A. flexuosus and G. lepidota cultures.

2.3.6.3 A. flexuosus 1171(I)c, FM1(I)a, and MFDb

A. flexuosus isolates 1171(I)c and FM1(I)a show a strong antigenic similarity with each other as well as with the reference strains R. meliloti ATCC 9930 and R. leguminosarum NC 128G53 and the native isolate G. lepidota V34(1). A. flexuosus 1171(I)c also appears to be related to R. trifolii and another G. lepidota isolate, V12(1)a. These relationships indicate that A. flexuosus isolates 1171(I)c and FM1(I)a should be affiliated with the fast-growing group of rhizobia, as well as the group containing the isolates of A. flexuosus and G. lepidota. Since A. flexuosus isolate MFDb was reciprocally adsorbed with R. leguminosarum NC 128G53, it, too, should be affiliated with the fast-growing rhizobia.

2.3.7 *Astragalus crassicarpus*

2.3.7.1 *A. crassicarpus* ACP2 and 6TS

Astragalus crassicarpus isolates ACP2 and 6TS did not demonstrate reciprocal adsorption with any of the reference strains or isolates. The *A. crassicarpus* ACP2 homologous titer was, however, significantly reduced after adsorption with 6TS and *R. leguminosarum*.

2.3.8 *Astragalus cicer*

2.3.8.1 *Astragalus cicer* NC 9B5

Astragalus cicer NC 9B5 was unusual in that it did not cross-react during cross-agglutination testing, but later was reciprocally adsorbed by three reference strains, one *G. lepidota* isolate, and one *A. flexuosus* isolate. This information would suggest that the *A. crassicarpus* isolate ACP2 and *A. cicer* strain NC 9B5 should also be included in the fast-growing group of rhizobia that encompasses isolates of *A. flexuosus* and *G. lepidota*.

2.3.9 Petalostemon purpureum

2.3.9.1 P. purpureum MFP

Petalostemon purpureum MFP did not display any reciprocal adsorption pattern with any of the reference strains or native isolates, but like previous examples, the homologous titer was reduced by adsorption with R. meliloti ATCC 9930 and A. crassicarpus ACP2. It would be premature to draw any conclusion about the classification of this isolate from this information.

2.3.10 Miscellaneous

2.3.10.1 A. tumefaciens PSI

Agrobacterium tumefaciens PSI did not show reciprocal adsorption with any other culture. The homologous titer of A. tumefaciens was equally reduced after adsorption with either R. leguminosarum NC 128G53 or A. flexuosus MFDb. Any classification of A. tumefaciens with relation to the Rhizobium species would not be possible with these data.

2.4 Serological relationships of cultures isolated from the same locale

The predominance of a certain serotype within a region, as well as serotypes related to the dominant one, have been observed in R. japonicum studies (11). In the present study, there was a similarity in the cross-agglutination and adsorption patterns of the organism pairs G. lepidota WR1a(2) and WR3a(2); G. lepidota V12(1) and V34(1); A. flexuosus N1(2)YE and N2(3)YE; and A. flexuosus 1171(I)c and FM1(I)a. In each case, both organisms were isolated from the same plant species within the same region. The cross-agglutination and adsorption patterns of A. flexuosus N1(2)YE and N2(3)YE are so similar that they appear to be the same serotype. The two pairs of G. lepidota isolates and the A. flexuosus isolate pair appear to share many antigenic components, but are not serologically identical to each other.

The ability to isolate the same or nearly identical native legume serotypes from the same plant species within the same geographical location is significant. It strengthens the data supporting the relationship of the native legume isolates to the recognized species of Rhizobium and suggests the existence of a predominant organism (serotype) as determined by the

native legumes of the area and the soil conditions within a certain locale.

A. flexuosus isolates N1(2)YE and N2(3)YE had little serological reactivity with G. lepidota isolate N12(b), and the same was true of G. lepidota cultures WR1a(2) and WR3a(2) and A. flexuosus WR1(I)c. The lack of cross-reactivity between the native legume cultures isolated from the same region but from different plant species further supports the specific legume-isolate symbiont relationship.

3. Identification of bacteroids by indirect fluorescent antibody technique

3.1 Standardization of IFA method

The indirect fluorescent antibody technique is a sensitive means of detecting antigen-antibody reactions. After the appropriate antisera had been adsorbed and proven monospecific, they were utilized as reagents in indirect fluorescent antibody microscopy. The dilution of antibody required for maximum fluorescence was determined using stock cultures of the appropriate isolate. After the conditions of the technique had been standardized, the bacteroids from the nodules of greenhouse-infected plants were tested.

This test was developed by reacting the homologous antisera of six reference strains or isolates against both the laboratory-grown cultures and plant nodule bacteroids. It would have been desirable to include more organisms reactive with the adsorbed sera, but such bacteroid-containing nodules were not available.

3.2 Double-blind study results

Utilizing the indirect fluorescent antibody technique, a double-blind study was conducted using all combinations of the six different monospecific antisera and bacteroids from nodules of greenhouse-infected plants. The six strains or isolates and host plants tested were:

1. *Trifolium pratense*: *R. trifolii* 127P17
2. *Medicago sative*: *R. meliloti* 9930
3. *Glycyrrhiza lepidota*: *Rhiz. sp.* WRla(2)
4. *Glycyrrhiza lepidota*: *Rhiz. sp.* (2)
5. *Trifolium pratense*: *Rhiz. sp.* L12(1)
6. *Astragalus cicer*: *Rhiz. sp.* 9B5

This procedure was developed to provide a rapid means of identifying the native legume infecting organisms. A correct identification was made for two of the six isolates, *G. lepidota* WRla(2) and *A. cicer* NC 9B5. This procedure was complicated by the frequent

occurrence of a strong background fluorescence which resulted in false positive identifications. A possible explanation for the occurrence of these false positives could be the presence of unadsorbed cross-reactive antibodies. The presence of any unadsorbed cross-reactive antibody might permit the antiserum to bind to a heterologous isolate. Since the indirect fluorescent antibody technique is a more sensitive means of detecting antigens than agglutination, antisera with no detectable agglutinins may still have reactive antibody detectable by indirect fluorescent antibody microscopy. In future studies, completeness of the adsorptions should be tested using the indirect fluorescent antibody procedure.

A second explanation for the background fluorescence might be the cross-reactivity of the antibody with plant lectins. Lectins, phytohemagglutinating glycoproteins, are felt to play a vital role in the bacteria-plant recognition required for successful rhizobium-legume symbiosis. It is now accepted that lectins may act as a cross-bridge between cross-reactive antigens on rhizobial cell walls and root hair surfaces. This suggests that lectin release may be a prerequisite for the adsorption of bacteria to legume roots, and partially explains the rhizobial

recognition process and host specificity between Rhizobium and legumes (13). This theory has, however, been complicated by the demonstration that some rhizobia do not bind with the lectin extracted from their host plant while other rhizobia bind with lectins from plants that they do not nodulate (13). During the indirect fluorescent antibody testing, it is possible that lectins may have been associated with the root nodule material. Their presence may have been responsible for the binding of cross-reacting antibody, resulting in a false positive test.

During the standardization of this technique, controls run on normal (non-nodule) plant root material were satisfactory. This observation indicates that either some substance present after nodulation is responsible for the false positives or that cross-reactive antibodies remained after adsorption. At this point it is impossible to eliminate either possibility but future studies should address themselves to these difficulties.

SUMMARY AND CONCLUSIONS

This investigation accomplished two broad objectives: 1) to further identify and define serological relationships among the Rhizobiaceae and 2) to develop a sensitive method for the identification of rhizobia from native legume root nodules. In addition, this study also verified the results of previous serological studies on the rhizobia reference strains. The objectives were met using serological techniques to expand information previously acquired on many of the native isolates and to correlate this with studies using reference strains. In conjunction with Eide's work (18, 19), this project offers valuable information on the potential classification of the rhizobia isolated from native legumes.

Without more extensive adsorption testing, it is impossible to define the minimal antigenic composition of the rhizobia tested. However, valuable information about the serological relationships among these rhizobia tested was obtained. There were strong antigenic relationships among rhizobia isolated from a plant species in the same location. Examples of this were: Glycyrrhiza lepidota WR1a(2) and WR3a(2);

Astragalus flexuosus N1(2)YE and N2(3)YE; and Astragalus flexuosus 1171(I)c and FM1(I)a. This serological similarity was unique to the isolates of each plant group as there was little or no cross-reactivity with the rhizobia isolated from the different plant species in the same region. For instance, A. flexuosus N1(2)YE and N2(3)YE were not strongly related to G. lepidota and G. lepidota isolates WR1a(2) and WR3a(2) were not serologically related to A. flexuosus isolate WR1(I)c. This illustrates the concept of nodulation specificity and confirms the existence of specific symbiotic pairing of native legumes and rhizobia. The specificity of this symbiotic relationship has been abundantly documented with the commercially important rhizobia and legumes.

A classification of rhizobia into three groups based on serological studies, biochemical and growth characteristics, and other data proposes the following groups: 1) the fast-growers - R. trifolii, R. phaseoli, and R. leguminosarum; 2) the slow-growers - R. japonicum, R. lupini, and the cowpea rhizobia; and 3) R. meliloti.

In general, the reference strains exhibited the same serological reactivity reported in other studies. Due to the strong cross-agglutination and

reciprocal adsorption reactions demonstrated by R. meliloti strains with R. leguminosarum, R. trifolii and R. phaseoli strains, results of this study cannot support the complete separation of the fast-growing group from R. meliloti.

Preliminary conclusions were obtained from this study on the relationships of native legume isolates to reference strains representative of these three groups. First, it was noted that two representatives of both of the type species, R. leguminosarum NC 128G53 and R. meliloti ATCC 9930 have strong serological similarities with the rhizobia isolated from many of the different species of native legumes. Evidence for a common antigenic component includes the reciprocal adsorption pattern displayed by these strains in this study, and supports the cross-agglutination results previously reported between these species. It is possible that these two reference strains, R. leguminosarum NC 128G53 and R. meliloti ATCC 9930, could possess an immunodominant component common to rhizobia isolated from many plant species. The extensive serological cross-reactivity of the rhizobia makes this conjecture impossible to prove with the available data.

G. lepidota isolates seemed to share many of

the same antigenic components with the fast-growers, R. meliloti ATCC 9930 and isolates of A. flexuosus and A. cicer. Thus, it would appear that the rhizobia isolated from the native legumes G. lepidota, A. flexuosus, and A. cicer are strongly related serologically to each other as well as to both the fast-growers and R. meliloti. It is not possible to classify these isolates any further with the information currently available. Other studies, including DNA base homology, DNA base composition, and further serological studies, are needed in order to complete the classification of these isolates.

Rhizobia isolated from the native legumes A. crassicarpus, P. purpureum, and the A. tumefaciens culture were not very reactive with the antisera prepared against the isolates of other native legumes or the reference strains. Placement of these isolates into one of the three groups would be premature. It is not unlikely that additional studies of native legumes isolates will mandate the formation of new groups in order to better classify these organisms.

The existence of extensive serological cross-reactivity between the three groups and among those groups and the native legume isolates used in this study shows that classifying the Rhizobium into only

three groups based on serological, biochemical, genetic and growth characteristics may not be possible. Perhaps, after more comparisons are completed, a classification scheme will include additional groups based on growth characteristics, and then these groups will be further subdivided into serological groups. The same serological sub-groups may be found in any or all of the larger groups, since this kind of classification scheme would account for organisms that have different growth and biochemical traits but share antigens. Such a scheme would explain the cross-reactivity among the reference strains and native legume isolates.

Additional studies are required to refine the method of serological identification of native rhizobia. The indirect fluorescent antibody technique shows great promise, but the problems associated with false positive identification must first be overcome. The wide-ranging applications of this method which would then be possible promises to make it a potentially valuable tool in future studies of rhizobia, and, therefore, warrants further development.

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APPENDIX I. *Rhizobium* Root Nodule and *Agrobacter* Cultures Studied and Their Source

Species of Plant Isolated From	Culture	Source
Trifolium pratense	*Rhizobium trifolii* ATCC 14480	Reference strain, American Type Culture Collection
Trifolium pratense	*Rhizobium trifolii* NC 127P17	Reference strain, Nitragin Company
Phaseolus vulgaris	*Rhizobium phaseoli* ATCC 14482	Reference strain, American Type Culture Collection
Phaseolus vulgaris	*Rhizobium phaseoli* NC 127K19	Reference strain, Nitragin Company
Medicago sativa	*Rhizobium meliloti* ATCC 9930	Reference strain, American Type Culture Collection
Medicago sativa	*Rhizobium meliloti* NC 102F65	Reference strain, Nitragin Company
Pisum sativum	*Rhizobium leguminosarum* ATCC 10004	Reference strain, American Type Culture Collection
Pisum sativum	*Rhizobium leguminosarum* NC 128G53	Reference strain, Nitragin Company
Glycyrrhiza lepidota	WR1a(2)	White River, South Dakota
Glycyrrhiza lepidota	WR3a(2)	White River, South Dakota
Glycyrrhiza lepidota	V12(1)a	Volga, South Dakota
Glycyrrhiza lepidota	V34(1)	Volga, South Dakota
Glycyrrhiza lepidota	N12b(2)	Norbeck, South Dakota
Glycyrrhiza lepidota	MFY	Yankton, South Dakota

APPENDIX I. (cont'd.)

Species of Plant Isolated From	Culture	Source
Trifolium pratense	L12(1)	Sinai, South Dakota
Astragalus flexuosus	N1(2)YE	Norbeck, South Dakota
Astragalus flexuosus	N2(3)YE	Norbeck, South Dakota
Astragalus flexuosus	WR1(I)c	White River, South Dakota
Astragalus flexuosus	1171(I)c	Highway 117, Black Hills, South Dakota
Astragalus flexuosus	FM1(I)a	Flag Mountain, South Dakota
Astragalus flexuosus	MFDb	Toronto, South Dakota
Astragalus crassicarpus	ACP2	Sioux Prairie, South Dakota
Astragalus crassicarpus	6TS	Antelope Range Station, South Dakota
Astragalus cicer	NC 9B5	Obtained from Nitragin Company
Petalostemon purpureum	MFP	Flandreau Prairie, South Dakota
Tomato plant	*Agrobacter tumefaciens* PSI	Obtained from Plant Science Department, SDSU

APPENDIX IV. Buffers Used in Globulin-Labeling Procedure

1. Carbonate-bicarbonate buffer

   a. Carbonate Solution

Na_2CO_3	5.3 g
Distilled water	100 ml

   b. Bicarbonate Solution

$NaHCO_3$	4.2 g
Distilled water	100 ml

   Add 17 ml carbonate solution to 100 ml bicarbonate solution to attain a pH of 9.0.

2. Phosphate-buffered saline

   a. Phosphate Solution A

Na_2HPO_4	1.4 g
Distilled water	100 ml

   b. Phosphate Solution B

NaH_2PO_4	1.4 g
Distilled water	100 ml

   Add 84.1 ml phosphate solution A to 15.9 ml phosphate solution B. Add 8.5 g NaCl, dilute to one liter volume with distilled water.